ENZYMATIC ISOLATION OF AN OSTEOBLASTIC PREPARATION SUITABLE FOR THE INVESTIGATION OF CELLULAR CALCIUM TRANSPORT

by

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A dissertation submitted to the faculty of the
University of Utah in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Department of Pharmacology

University of Utah

June, 1976

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

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ACKNOWLEDGMENTS

I wish to thank all members of the Department of Pharmacology for their contributions to my graduate education. I am also indebted to Dr. Ewart A. Swinyard and the College of Pharmacy for making this training possible.

Appreciation is expressed to Drs. Webster S. S. Jee, William K. Nichols, Lester M. Partlow, Donal J. Reed and Dixon M. Woodbury for serving on my thesis committee. I am particularly obliged to Dr. Woodbury for his helpful guidance and to Drs. Jee and Donald B. Kimmel for their advice in histological matters.

I wish to thank Ken and Virginia Olson for allowing me to share their home during the preparation of this manuscript and Elaine Frederickson and the secretarial staff for typing my dissertation in final form.

I would also like to thank Dr. James W. Gibb for his valuable friendship and council during the past four years.

Finally, I am eternally grateful to my wife, Dolores, whose love and patience were of constant support throughout my graduate training, and to our parents, Stanley, Kathryn, Gordon and Esther for their continuous encouragement and assistance.

This work was supported by USPHS grants GM 00153 and DE 00252.

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ABSTRACT

A microanatomical study of the immature rat calvarium was performed in order to develop a method for the isolation of osteoblasts by enzymatic means. Although generalized osteogenesis was evident in fetal rats, differential growth patterns were observed beginning at 19 days *in utero*. Considerable portions of the endocranial periosteal bone surface were lined by flattened, less active cells; discrete areas also contained multinucleated osteoclasts. Cell counts of whole calvaria revealed that 1/5 of the total cell population were osteoblasts, most of which were located in the central portions of the frontal and parietal bones. Prior excision of these segments permitted the subsequent removal of virtually all periosteal tissues. Cleaned 19-day fetal bones, incubated in crude collagenase for 2 hr, released about 40,000 cells/calvarium, consisting of 85-90% osteoblasts and lesser amounts of connective tissue and bone marrow elements. Because of the relatively small sizes of most extraneous cells, purity on a cell volume basis was approximately 95%.

Measurements of the calcium content in isolated osteoblasts were found to be adversely affected by 3 major factors, including: 1) the presence of calcium-containing particles in the crude collagenase

preparation utilized for cell isolation; 2) a calcium-accumulating effect of prolonged collagenase exposure on cell isolates; and 3) the existence of pronounced osseous fragment contamination. These detrimental influences were eliminated respectively by: 1) filtration of collagenase solutions prior to bone incubation; 2) reduction of the collagenase exposure time; and 3) utilization of a mild acid incubation to solubilize osseous fragment mineral. These procedures, in concert, reduced the apparent osteoblastic calcium concentration from approximately 160 to 10 mM. The latter value should be considered only a maximal limit at this time since it is not yet clear if mild acid exposure removes all osseous calcium deposits.

PART I

ENZYMATIC ISOLATION OF OSTEOBLASTS FROM FETAL RAT CALVARIA

ABSTRACT

A microanatomical study of the immature rat calvarium was performed in order to develop a method for the isolation of osteoblasts by enzymatic means. Although generalized osteogenesis was evident in fetal rats, differential growth patterns were observed beginning at 19 days in utero. Considerable portions of the endocranial periosteal bone surface were lined by flattened, less active cells; discrete areas also contained multinucleated osteoclasts. Cell counts of whole calvaria revealed that 1/5 of the total cell population were osteoblasts, most of which were located in the central portions of the frontal and parietal bones. Prior excision of these segments permitted the subsequent removal of virtually all periosteal tissues. Cleaned 19-day fetal bones, incubated in crude collagenase for 2 hr, released about 40,000 cells/calvarium, consisting of 85-90% osteoblasts and lesser amounts of connective tissue and bone marrow elements. Because of the relatively small sizes of most extraneous cells, purity on a cell volume basis was approximately 95%. It is predicted this preparation will be useful in the investigation of certain aspects of osteoblastic function.

INTRODUCTION

With the recognition that bone cells are directly responsible for the regulation of skeletal-dependent calcium homeostasis (11, 19), attention has focused on what cellular mechanisms may be involved (10, 20, 27, 30). Histochemical and electron microscopic techniques have shown that certain bone cells, notably osteoblasts and osteocytes, can sequester or release calcium in response to appropriate hormonal agents (16). Quantitative information, however, concerning cellular calcium movements is limited. Although in vitro investigations of fetal bone tissue have been invaluable in establishing the concept of a "functional bone membrane" separating bone salt from the normal extracellular space (26), similar studies have been less useful in evaluating cellular calcium exchange because of the relatively enormous deposits of mineral in the attendant ossified tissues (18). One promising approach to this problem involves the separation of bone cells from fetal rat calvaria by enzymatic digestion (3, 5, 22, 28). Cells isolated in this manner have been successfully cultured (3, 22) and are known to be affected by parathyroid hormone and by calcitonin (6, 23, 28). A limitation, however, to studies of this kind relates to the diversity of cell types present in such preparations. Experi-

ments with partially segregated bone cells derived from fetal (32) and from adult (29) rat tissues indicate that several classes of cells in bone respond quite differently to treatments affecting calcium regulation. Studies of cell populations with unknown compositions are therefore of questionable value, and it appears necessary that such mixtures will have to be separated into their component subgroups before a clear understanding of the cellular control of skeletal mineral metabolism can be achieved.

The investigation reported herein was an attempt to isolate pure osteoblasts from fetal rat calvaria by combining the method of Peck et al. (22) with a more rigorous dissection technique aimed at eliminating the chief sources of connective tissue contamination. A thorough histological assessment of calvaria at various stages of development and of the cells derived therefrom was necessary in order to attain that objective.

MATERIALS AND METHODS

Calvaria used in this study were taken from Sprague-Dawley rats ranging in age from 17 days *in utero* to 40 days post-partum. The braincase segment normally removed for study is indicated in Fig. 1. The incubation medium was Earle's balanced salt solution (Grand Island Biological Co.) modified by a reduction of the calcium concentration to 1.25 mM and the addition of 200 mg/ml bovine serum albumin (Fraction V; Miles Laboratories, Inc.).

Whole tissue procedures

Light microscopy. Rats were sacrificed by decapitation, and the calvaria were quickly excised, rinsed in ice-cold Earle's medium and fixed in Millonig's phosphate-buffered formalin (pH 7.0) for 48 hr. The calvaria were decalcified in 14% EDTA (pH 7.0) for 72 hr and stored in 70% ethanol for at least 24 hr. All tissues were embedded in methyl methacrylate according to the method of Kimmel and Jee (15). Coronal and sagittal sections (2.5μ thick) were cut with a Jung Model K microtome and affixed to gelatin-coated slides. The embedding plastic was removed by acetone, and the slides were stained with Mayer's hematoxylin and eosin. Tissues taken at various stages of the bone cell isolation procedure were prepared for

microscopic study in a similar manner, except that decalcification in EDTA was often omitted.

Scanning electron microscopy. Tissues were fixed in a cacodylate-buffered (pH 7.4) solution of 1% osmic acid for 1 hr and quickly air dried. The tissues were then coated with evaporated films of carbon and gold. Specimens were viewed at an angle of 45° with a Cambridge Stereoscan Mark 2 operated at an accelerating voltage of 20 kV.

Cell mapping. Differential cell counts were performed on 10 specified regions of histological sections prepared from 19-day fetal calvaria (Fig. 2). All cells in a 250μ long coronal (sites 1-2, 8-9) or sagittal (sites 3-7, 10) segment of tissue were counted per site in each of 4 fetal rats. Because individual sampling volumes were of uniform surface dimensions (2.5 x 250μ), the cell counts provided relative indices of cellularity per unit tissue surface area.

Total surface areas were estimated from calvaria in situ. Tracings of calvarial tissue normally taken for study (Fig. 1) were made on paper with an overhead projector. The tracings were cut into pieces representing the 5 major portions of the calvarium including: 1) the central areas of the frontal and parietal bones (indicated by the dashed lines in Fig. 2); 2) the remaining peripheral portions; and 3) the sutures. The segregated pieces were weighed separately, and the percentages of total paper weight were used as

measures of % total surface area. Since the part of the calvarium used in this work was fairly flat, error because of cranial curvature was deemed minimal.

By assuming that cell counts for the sites chosen were representative of the areas in which they were located, the total percentage of osteoblasts in the calvarium was calculated as follows:

$$\frac{\%\ \text{osteoblasts}}{\text{calvarium}} = \frac{\Sigma(\text{osteoblasts/unit area})\ (\%\ \text{total surface area})}{\Sigma(\text{total cells / unit area})\ (\%\ \text{total surface area})}$$

For areas including more than one site, mean values were used. Sites 5 and 10 were disregarded in these calculations, site 5 because of compression of the coronal suture following calvarial excision and site 10 because it was not a representative sample.

Isolated cell procedures

Bone cell isolation. Pregnant rats were anesthetized with sodium pentobarbital injected subcutaneously. Fetuses, normally 8-12/mother, were taken by cesarian section and perfused with ice-cold Earle's solution by cardiac puncture. Postnatal rats were anesthetized with ether prior to perfusion. Calvaria were removed and transferred to a watch glass containing Earle's solution (which was frequently replaced with fresh medium to maintain a constant pH of 7.4). With the aid of a dissecting microscope, the bones were cut out individually, leaving a wide margin of immature bone completely surrounding the major suture spaces (Fig. 2). The periosteum

was then carefully stripped away. (Cells derived from bones handled in this manner constituted the osteoblast preparation.) The dissected bones were placed into siliconized flasks (approximately 40 bones/flask) containing 6 ml Earle's solution with 2.5 mg/ml crude collagenase (CLS II; Worthington Biochemical Corp.) The medium was thoroughly gassed with 95% O_2 and 5% CO_2, and the flasks were then tightly stoppered, transferred to a water bath shaker and incubated at 37°C and 100 oscillations/min for 2 hr. To aid in the identification of isolated cells, the dissected periosteal tissues were incubated initially in separate flasks instead of being discarded. On occasion, whole calvaria with the periosteum removed but the sutures intact were also used for comparison purposes. (Cells released from the last source are referred to collectively as the mixed bone cell preparation.) After incubation, the cell suspension was decanted, and the bones were swirled several times with collagenase-free medium. The solutions were combined and passed through nylon bolting cloth (35μ mesh NITEX; Tobler, Ernst and Traber, Inc.) to trap large bone fragments and other debris. The filtrate was then centrifuged at 400g to pellet the cells. After several washings to remove any remaining collagenase, aliquots of the final suspension were taken for cell counting and viability determinations. Cells were routinely counted in a modified Levy chamber with phase contrast; viability was assessed at the same time by nigrosin exclusion (13).

Light microscopy. Isolated cells were prepared for microscopic observation with Wright's stain. The cells were centrifuged at 400g and resuspended in 30% fetal calf serum. Smears were made on washed coverslips either by hand or with a Plat sample spinner. The cells were then quickly air dried and stained. At times, cells were prepared for staining by allowing them to attach onto collagen-coated plastic petri plates (Falcon Products; Becton, Dickinson and Co.). This minimized mechanical damage to cells observed in most smears (normally ranging from 5-15%); however, staining was inferior with this method, and it served only to demonstrate that cell disruption was due to smearing and not to the isolation procedure. Alkaline phosphatase activity of the osteoblast preparation was assessed qualitatively by the method of Kaplow (14). Morphology of living cells was examined by phase and Nomarski interference contrast.

Cell identification. Differential counts were performed on osteoblast preparations, on mixed bone cells and on isolates derived from whole calvaria. Damaged and non-nucleated cells were noted separately. Criteria for identification in osteoblast preparations were the following: 1) Osteoblasts were defined as large (15-20μ) basophilic cells with eccentrically located nuclei, usually oval or circular in shape and possessing one or more nucleoli each. The bluish-purple cytoplasm was mottled in appearance and displayed a

central clear zone of reduced basophilia. 2) Cells were classified as lymphoid-like by their small size (5-10μ), often intensely hyperchromatic nuclei and very scanty cytoplasm. 3) The myeloid category consisted of cells that could easily be identified as such by the presence of azurophilic or (at later stages of development) specific granules and by their characteristic nuclear patterns (31). Myeloid cells were used to indicate the definite presence of bone marrow in the various cell preparations. 4) "Other" cells included the rest of the hematopoetic elements of bone marrow plus an admixture of mesenchymal cells.

Besides the lymphoid-like and osteoblast categories, mixed bone cells and populations derived from whole calvaria were classified into 2 other major groups according to descriptions presented by Ham (8). The active fibroblast was a large cell (20-30μ) with moderately basophilic cytoplasm, characteristically vacuolated in appearance. The nucleus was centrally placed, oval or circular in shape, and contained one or more nucleoli. Membrane edges appeared folded in all cells. The macrophage was typically smaller in size (15-20μ) with a less spongy-looking cytoplasm. The nucleus was more condensed than that observed in the fibroblast and was indented or kidney-shaped. Azurophilic granules were often observed, but their presence was not necessary for identification. Cells not fitting any of these 4 categories were labelled "others" and included mast cells, marrow elements and

cells transitional in morphology between lymphoid-like cells and osteoblasts, fibroblasts and macrophages.

RESULTS

Anatomy of the rat calvarium

The calvarium is comprised of 5 bones (the interparietal and the paired frontal and parietal bones) and 4 major sutures (the metopic, sagittal, coronal and anterial lambdoidal sutures) (Fig. 1). Because the interparietal bone proved to be totally unsuitable for the isolation of osteoblasts, it was usually omitted in dissection entirely and will not be considered further. At every age studied, the bones comprising the immature calvarium exhibited marked heterogenous morphologies. This is best explained by the overlapping of various stages in intramembranous bone formation within each tissue.

Development. The present study confirmed the sequence of events in the early phase of growth in the calvarium as described by Moss (17). Ossification begins when clusters of mesenchymal cells within the neurocranial capsule differentiate into osteoblasts. Spicules of bone, the primary trabeculae defined by Nobeck (21), soon form and spread radially from the 4 centers of ossification corresponding to the paired frontal and parietal bones. As growth proceeds, secondary trabeculae interconnect with the primary spicules, and the bone assumes a spongy framework containing numerous spaces filled with soft tissue. Appositional growth on all surfaces fills in the network,

leaving a solid plate of bone covered on both periosteal surfaces by a continuous layer of osteoblasts. Eventually, through differential formation and resorption, the adult form consisting of 2 walls of bone enclosing a cancellous diploe is realized.

Histology. Examination of the immature rat braincase revealed that various phases of bone formation were coexistant within different portions of the same bone. In the 19-day fetal calvarium, which is diagrammed in Fig. 2 and will be described in detail, the outermost edges of the bones consisted of primary trabeculae, still maintaining the radial orientation observed in earlier stages of development. Between this peripheral layer and the central, solid plate of bone was another zone where secondary trabeculation had occurred but where connective tissue spaces between the spicules had not yet been obliterated. Although the general features of the frontal and parietal bones were similar, several important differences were noted.

The parietal bone of the 19-day fetus was fairly uniform in appearance. The central portion of bone was surrounded by a highly organized periosteum (Fig. 3a, 7a). For the most part, the cells lying adjacent to the bone were large osteoblasts with eccentric nuclei and prominent juxtanuclear clear areas corresponding to well-developed Golgi bodies. In certain areas of the endocranial periosteal surface, however, some cells appeared flattened, perhaps beginning the transformation into the adult form of lining cell. Just outside the osteo-

blastic layer were cells which seemed transitional in nature. These cells, presumably pre-osteoblasts, were commonly seen in young bones but were less obvious in older tissues. They were intermediate in appearance between osteoblasts and the more prevalent fibroblasts. In parietal bones, fibroblasts consisted mostly of cells with little cytoplasm and flattened, hyperchromatic nuclei. To distinguish these cells from larger, more active-looking fibroblasts, they will hereafter be referred to as fibrocytes.

The peripheral parietal bone was similar to the central region except that spaces between osseous spicules were evident (Fig. 3b). In addition, the bone was thinner, and osteoblasts with lining cell morphologies were less apparent. Periosteal tissues exhibited the same highly organized cellular framework. As with the rest of the parietal bone, osteoclasts were not observed in the 19-day fetus, and osteocytes, except for their location, were indistinguishable from osteoblasts.

One deviation from the typical parietal morphology was found in the most caudal region of the bone adjacent to the sagittal suture (Fig.3b). Portions of the falx cerebri were often left attached after calvarial removal. Furthermore, a bar of cartilage was noted extending from underneath the anterior lambdoidal suture and lying between the falx and the endocranial periosteum. Thought by Pritchard et al. to arise from the tectal region of the chondrocranium (25), the cartilagenous

rod was ephemeral in nature and disappeared soon after birth.

The microanatomy of the frontal bone could be distinguished from the parietal in several respects. Generally, the frontal bone was more developed at this age. The whole tissue was thicker, and areas of primary and interconnecting trabeculation were more circumscribed. Perhaps the greatest difference lay in the early formation of the ectocranial wall (Fig. 4). In peripheral areas where large holes still existed within the bony plate, spicules of bone were observed extending dorsally from the presumptive endocranial table. Osteoblasts were found in more developed regions layered between the vertical trabeculae, beginning to lay down new bone even before the spaces of the endocranial plate were closed (Fig. 6a). With the simultaneous initiation of resorption to complicate development (Fig. 5), the resultant bony framework was often complex (Fig. 6b). Finally, around the original center of ossification, ecto- and endocranial tables of bone were present, with appositional growth occurring primarily on the dorsal surface of each wall.

Besides exhibiting alterations in osseous formation, the frontal bone was also unique in its soft tissue organization. Instead of highly uniform layers of osteoblasts and fibrocytes, periosteal constituents were more loosely constructed with fewer but more variegated cells. In addition to fibrocytes, a number of cells were present that contained rounder nuclei and greater quantities of cytoplasm.

Although specific stains were not utilized, it was apparent that these cells consisted of several different types. Flattened osteoblasts (or lining cells) were more evident in the frontal bone, covering the developing diploe in addition to areas on the endocranial table. Multinucleated osteoclasts were also found along the endocranial periosteal surface (Fig. 5).

A final difference between the parietal and frontal bones was the relatively greater proportion of hematic elements present in the latter structure. Sinuses with large numbers of erythrocytes were seen in histological sections (Fig. 4b); myeloid cells were observed on occasion in the developing marrow spaces.

Generally, the 3 major sutures of the calvarium (the anterior lambdoidal having been removed along with the interparietal bone) were similar, composed of highly organized layers of connective tissue cells, most of which resembled fibrocytes. The coronal suture was exceptional only because it appeared pinched in excised calvaria. This caused the tissue to look somewhat thicker than normal in cross-section.

Cell mapping. Bone cell populations at specified regions of the 19-day fetal calvarium were counted in order to determine what tissues should be taken for enzymatic incubation and what should be discarded (Table 1). Results were consistent with the microanatomical observations. Comparison of the frontal (sites 2-4) and parietal (sites 6-8)

bones demonstrated that the frontal was somewhat more cellular per unit surface area, indicative of the greater thickness of that tissue. The percentages of osteoblasts and osteocytes were also higher, reflecting primarily the early formation of the ectocranial plate. Within each bone, the proportioning of cells was fairly constant with respect to location; peripheral areas, however, tended to have fewer cells (compare sites 2 and 4 with 3 and sites 6 and 8 with 7). Of the sutures (sites 1, 5 and 9), the coronal (site 5) had about 20% more cells per unit surface area. This was explained by the distortion present in that area. The anomalous results associated with site 10 reflected the presence of extra cells in the cartilaginous rod and in the falx cerebri.

With the values in Table 1 and estimates of the surface areas of the pertinent structures involved, calculations were made concerning the total percentages of cells in the rat calvarium (Table 2). About 1/2 of all osteoblasts were located in the central portion of the frontal bone, with most of the rest found in the body of the parietal. Sutures, which of course made no contribution to the osteoblastic pool, accounted for 1/5 of the total population. In all, it was estimated that 19% of all cells were osteoblasts.

Isolation of osteoblasts

Initial attempts at removing periosteal tissues without disturbing the osteoblastic layer were disappointing. Although portions could be

stripped off, complete removal was impossible, particularly around areas to which the periosteum was adherent (such as sutures). Since cell mapping indicated that the central portions of bone contained most of the osteoblasts, it was decided to separate them from the rest of the calvarium before attempting periosteal detachment. This procedure was successful (Fig. 5b, 7a,b). Even so, use of a dissecting scope was absolutely essential to avoid connective tissue contamination.

Incubation in collagenase released almost all cells remaining on the bone surface after dissection (Fig. 7c). Removal of osteocytes was not as effective, however. (Of 200 parietal lacunae examined, more than 2/3 contained cells.) About 95% of the isolated cells were viable as determined by phase brightness and by nigrosin exclusion. Total yield was usually around 40,000 cells/calvarium.

Influence of age. The question of what age rat to choose for routine cell isolation was considered. Although several investigators had reported using diversified ages (22, 28), the rapid calvarial growth during the prenatal period suggested that cell recovery might also vary with age. Accordingly, osteoblast preparations were made from rats ranging in age between 17 days *in utero* and 40 days post-partum. It was evident after Wright's staining that the highest percentages of osteoblasts were obtained from 18 and 19-day fetuses (Fig. 8). In older age groups, the osteoblast fraction fell rapidly, reaching a

nadir of 20% by 40 days. The 17-day group was not tabulated because it proved virtually impossible to remove the periosteum completely.

The most common contaminant in the osteoblast preparations studied (with the exception of the 18-day fetal isolates) was the lymphoid-like cell. This class contained several different cell types, all of which were small, highly basophilic cells with scanty cytoplasm (Fig. 9). These cells became more prominent with age; by 40 days, they accounted for over 1/2 of all cells isolated. Bone marrow contamination also increased with time. Myeloid cells first appeared at 19 days of gestation, consisting mostly of precursor forms. By 21 days (birth), mature granulocytes accounted for about 50% of the myeloid elements present, and similar proportions were observed thereafter. Most extraneous cells in 18-day fetal preparations were from periosteal sources. Relatively large numbers of active fibroblasts and macrophages were present in addition to a few lymphoid-like and mast cells. Although the periosteum could be completely removed from the calvarium at this stage of development, dissection was time-consuming. Because the same percentage of osteoblasts could be recovered from animals 1 day older with much less difficulty, 19-day fetal calvaria were selected for all subsequent cell isolation experiments.

Osteoblast purity. Alkaline phosphatase activities and interference contrast assessments provided measures of cell purity indepen-

dent of Wright's stain. Of 400 cells counted from a 19-day fetal osteoblast preparation, 96% stained heavily for alkaline phosphatase, 2% reacted moderately and 2% were negative. In all, 88% exhibited both high activity and the typical osteoblast morphology. The remaining 8% of the heavily stained cells were quite small and most probably included a significant fraction of lymphoid-like elements. Although less reliable than staining, interference contrast was useful in identifying osteoblasts in suspension (Fig. 10). Visualization of eccentric nuclei and characteristically stippled membrane surfaces permitted (along with cell diameter considerations) discrimination between osteoblasts and other cells.

In all, the percentage of isolated cells identified as osteoblasts varied from 85-90%. Calculated on a cell volume basis, purity was considerably better. Whereas osteoblasts in suspension have a mean diameter of 14μ (Yagiela and Woodbury, submitted for publication), the other cells present averaged only 10μ. Assuming a preparation contained 85% osteoblasts, purity on a cell volume basis would approximate 95%.

Comparison with other procedures. Both the major advantage and disadvantage of the present method are illustrated in Table 3. Incubation of whole calvaria in collagenase yielded considerably more cells than did the other 2 procedures listed. Cell release was apparently non-discriminatory since the percentage of osteoblasts

recovered was similar to that calculated for the intact calvarium. Using a dissection method commonly employed by others (5), a modest improvement in osteoblast purity was achieved at the cost of a reduction in cell yield. The mixed bone cell population was composed of equivalent fractions of osteoblasts, active fibroblasts, macrophages and lymphoid-like cells. The number of erythrocytes in mixed isolates from unperfused rats was also significant, approaching 5×10^5 cells/calvarium in heavily contaminated samples. In contrast to the osteoblast preparation, calculation of purity on a cell volume basis did not increase the osteoblastic contribution in mixed bone cells. Active fibroblasts, generally larger than osteoblasts, made up for the relatively small size of the lymphoid-like cells. As noted, the dissection technique employed for the isolation of osteoblasts markedly enhanced the percentage of osteoblasts reclaimed. To achieve this, however, it was necessary to accept a considerable drop in the number of cells recovered.

DISCUSSION

Although several previous reports mentioned the existence of variations within and between fetal rat calvarial constituents (1, 17), the marked osseous and cellular differences noted here have generally not been described. Moss, for example, recognized that bone near an origional ossification center was at any point further advanced than more peripheral tissues and that calvarial bones developed at different rates with respect to each other (17). He still believed, however, that osteogenesis was uniformly active on all bone surfaces until well after birth. This view of undifferentiated growth, subsequently subscribed to by de Angelis (4), may be true in the grossest sense, but it ignores the rich diversity observed at the microanatomical level, where relatively quiescent regions of bone lined by flattened cells may be juxtaposed to rapidly modelling surfaces covered with active osteoblasts or to areas undergoing osteoclastic resorption. With the exception of Pritchard, who referred to the cells as medullary osteoblasts (24), the existence of inactive-looking osteoblasts in the fetal rat calvarium has been largely ignored. Reports of osteoclasts on ventral surfaces of neonatal braincases have also generally not been considered (2, 33).

The cellular heterogeneity noted histologically underscored the requirement for an accurate method of cell classification to monitor isolation procedures. Although Wright's stain proved adequate for the identification of osteoblasts in mixed cell suspensions, recognition of some other types was more hazardous. One obvious side effect of enzymatically releasing cells from any tissue is that normal cellular configurations and spatial relationships are lost. Lining cells, for example, identified by their flattened appearance and by their position next to bone, become indistinguishable in suspension from fibrocytes and pre-osteoblasts. Differentiation from small lymphocytes is difficult, and the whole problem is compounded by the presence of erythrocytic precursors which, in immature rats, are also very similar in appearance (8). For convenience, and to avoid the necessity of special staining, these cells were combined into one category, and attention was focused on other types that could be recognized individually with Wright's stain. Accordingly, myeloid cells were used as bone marrow markers, and active fibroblasts and macrophages were relied upon to indicate periosteal contamination. It was known from previous work that granulocytes made up a significant percentage of rat bone marrow, both in the fetus and in the adult (8, 12). Examination of isolated periosteal cells indicated a homologous relationship held for large fibroblasts and macrophages.

Work with mixed bone cell preparations confirmed the general

impression of other investigators using enzymatic isolation that cells released from partially cleaned fetal calvaria were heterogenous (6, 9). It was somewhat surprising that stripping off most of the periosteum before collagenase digestion had little beneficial effect upon the percentage of osteoblasts recovered as compared with whole calvarial isolates. If all of the cells removed during dissection were connective tissue contaminants, over half of the remaining cells should have been osteoblasts. Apparently, large quantities of osteoblasts are detached along with the periosteum. This is especially true of the inactive-looking osteoblasts, since examination revealed that many more cells remain on the dorsal surfaces of calvaria after dissection than on the ventral. The present method is successful in reclaiming a high percentage of osteoblasts only because virtually all of the periosteal and sutural cells are removed before enzymatic incubation.

Although studies with mixed bone cells have demonstrated the possibility of investigating bone cellular physiology in the isolated state, such work will remain suspect insofar as extrapolation to osteoblasts is concerned until much more is known about the relative contributions of the various cells present in the problems under investigation. For example, a recent examination of mixed bone cells indicated that osteoblasts might contain considerable amounts of calcium (5). If calcium is in fact sequestered in osteoblasts,

according to our cell counts the true concentration in osteoblasts might be 4 times higher than actually reported. If, on the other hand, extraneous cells contributed substantially to the total calcium measured, then deductions concerning osteoblasts based on the mixed cell data would be meaningless. It is hoped that the present method will aid in distinguishing between such alternatives and help to provide a more accurate assessment of osteoblastic function.

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Fig. 1. Diagram of the fetal rat skull. Dashed line indicates the portion of the calvarium routinely taken for study.

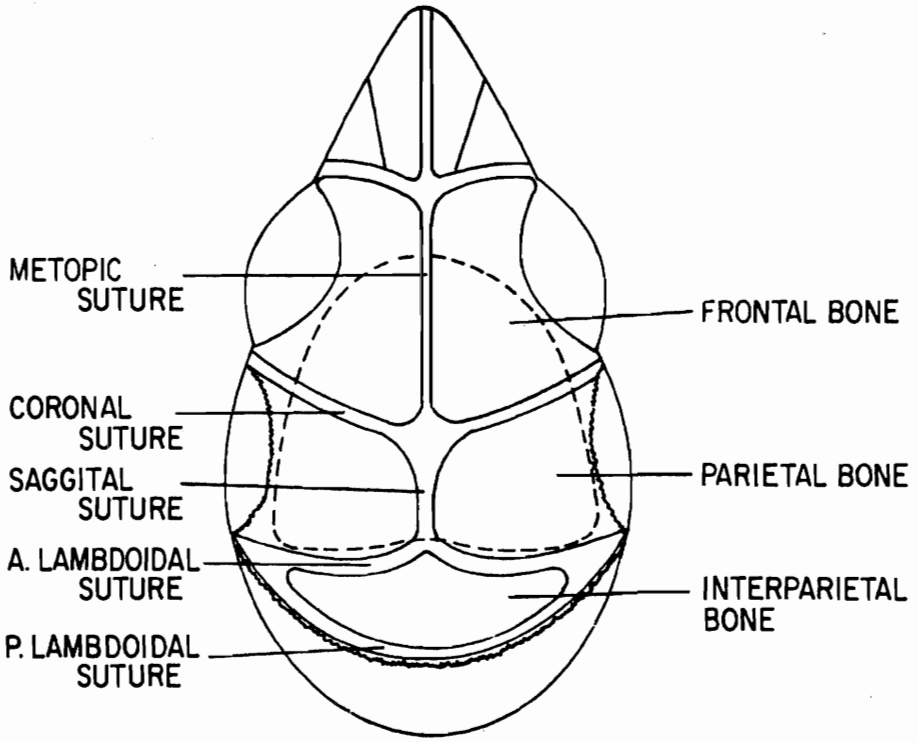

METOPIC SUTURE
CORONAL SUTURE
SAGGITAL SUTURE
A. LAMBDOIDAL SUTURE
P. LAMBDOIDAL SUTURE
FRONTAL BONE
PARIETAL BONE
INTERPARIETAL BONE

Fig. 2. Tracing of the excised fetal rat calvarium. Areas inside dashed lines are the portions of the frontal and parietal bones removed for osteoblast isolation. Areas of primary and secondary trabeculation in the 19-day fetus are indicated by white bars and stippling, respectively. The various sites chosen for histological examination and cell counting are numbered.

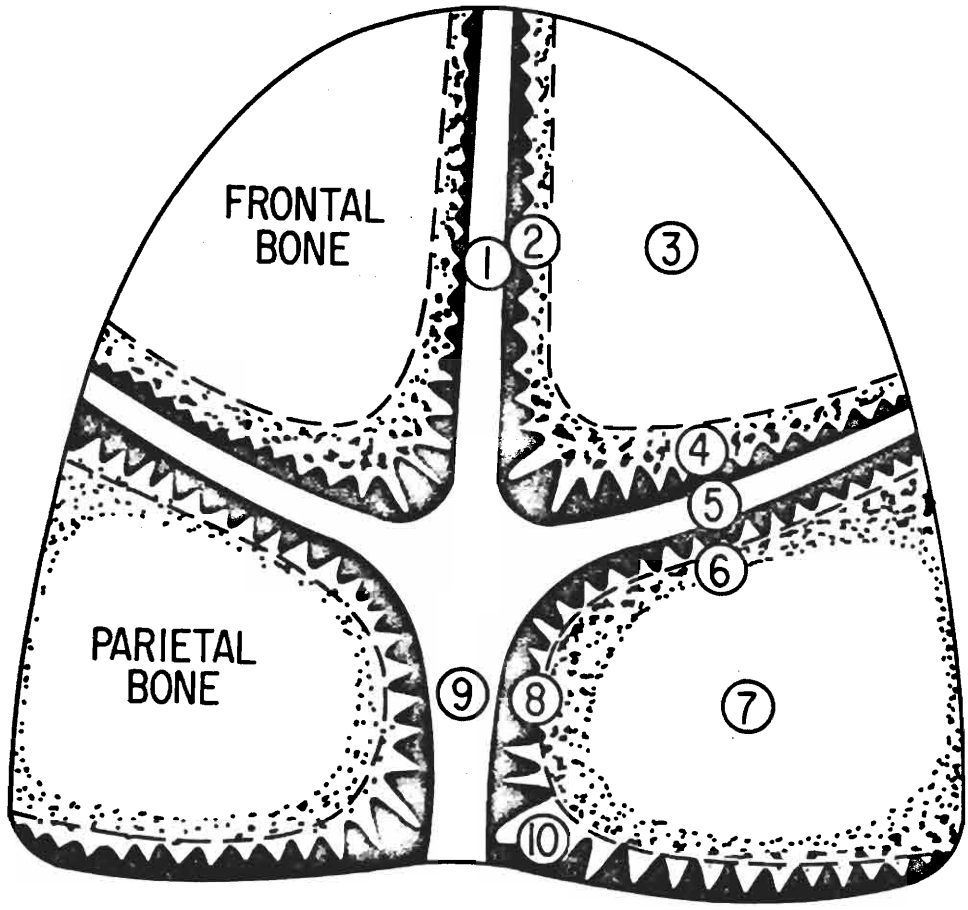
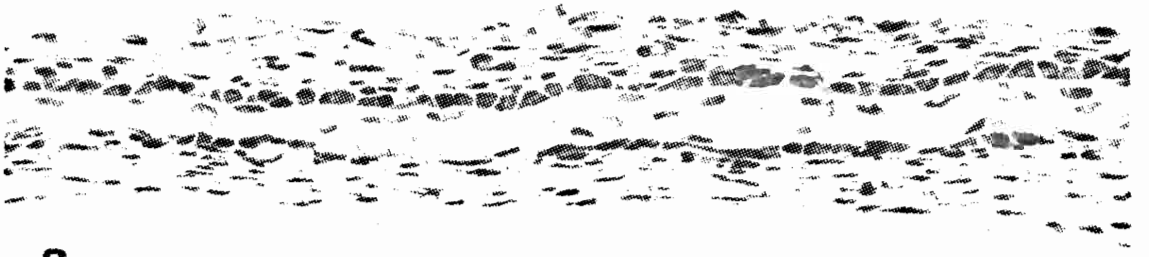

FRONTAL
BONE
PARIETAL
BONE
1
2
3
4
5
6
7
8
9
10

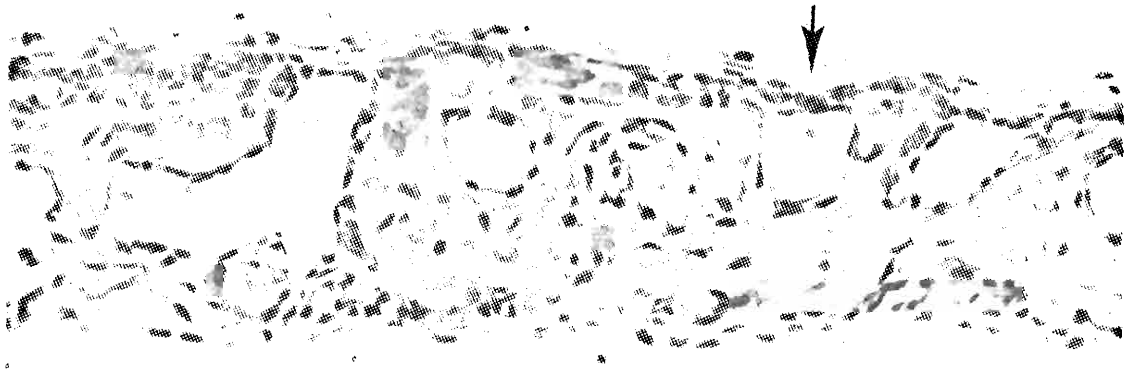
Fig. 3. Sagittal sections from the parietal bones of the 19-day rat fetus. H&E. X 250. a) Central area (site7) consisting of a solid plate of bone enveloped by a highly organized periosteum. b) Peripheral area (site 10) demonstrating typically incomplete plate formation and an atypical presence of cartilage (C), the falx cerebri (F) and a lateral segment of the superior sagittal sinus (S).



a

b

Fig. 4. Sagittal section from the frontal bone of a 19-day rat fetus. H&E. X 250. a) Peripheral area (site 4) including, on the right (towards the coronal suture), vertical trabeculae with osteoblasts bridging in between (arrow) and, in the center and left, the early development of the ectocranial plate. b) Central region (site 3) illustrating a more advanced development of the ectocranial wall. Note the erythrocytes in the sinus ventral to the endocranial bone (arrow).



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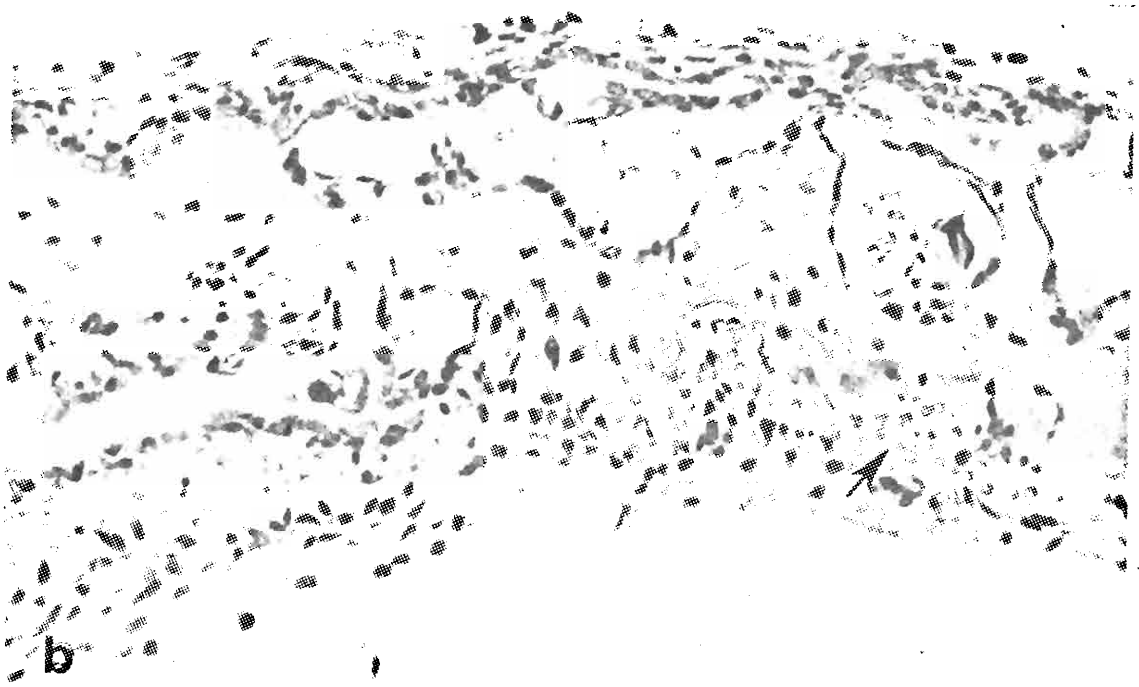

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Fig. 5. Photomicrograph of two osteoclasts (arrows) on the endocranial periosteal surface of the frontal bone. Active fibroblast (F). macrophage (M). H&E. X 930.

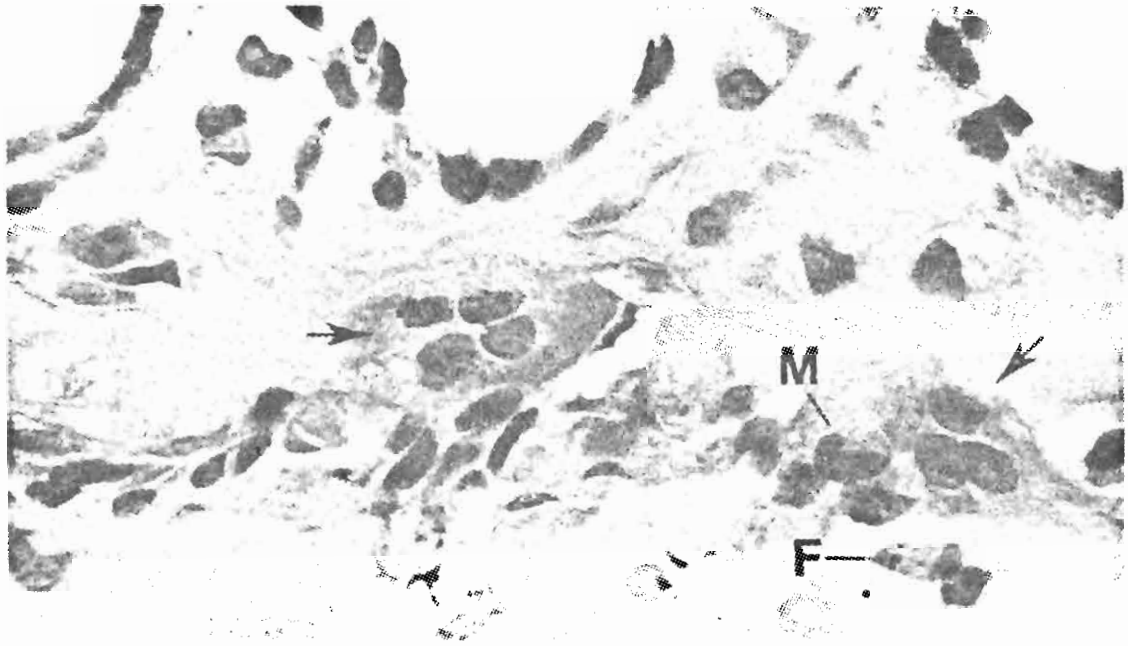
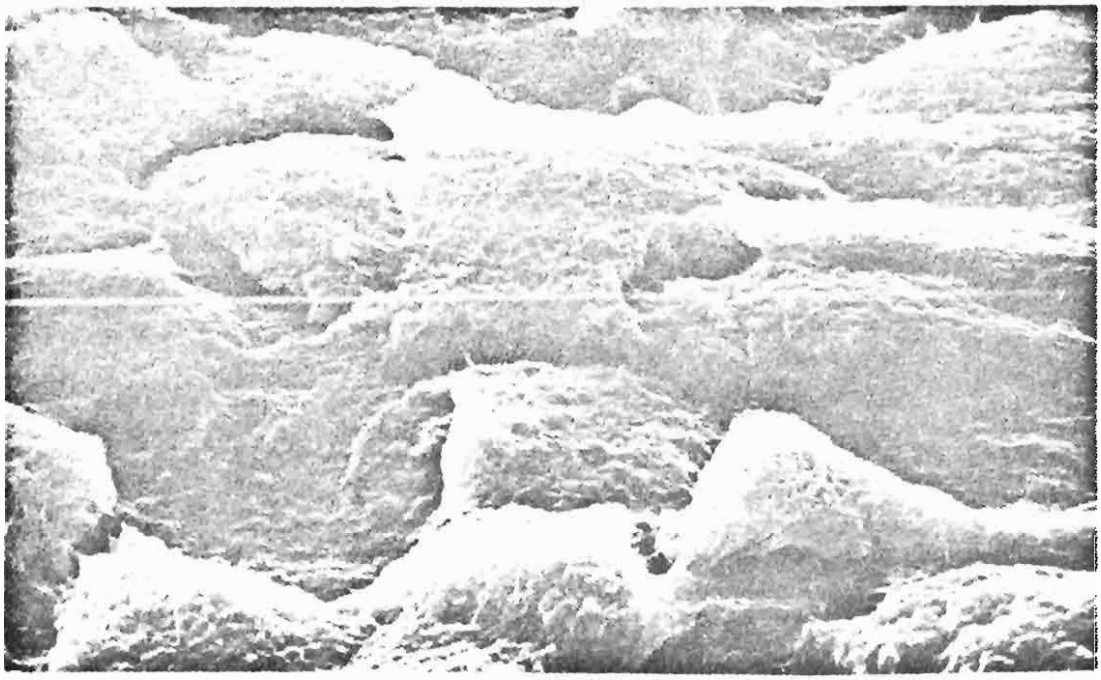

M
F

Fig. 6. Scanning electron micrographs. a) Dorsal view of the parietal bone showing numerous osteoblasts left after periosteal removal. X 2200. b) Ventral surface of the frontal bone after periosteal detachment demonstrating a complex trabecular arrangement. X 550.



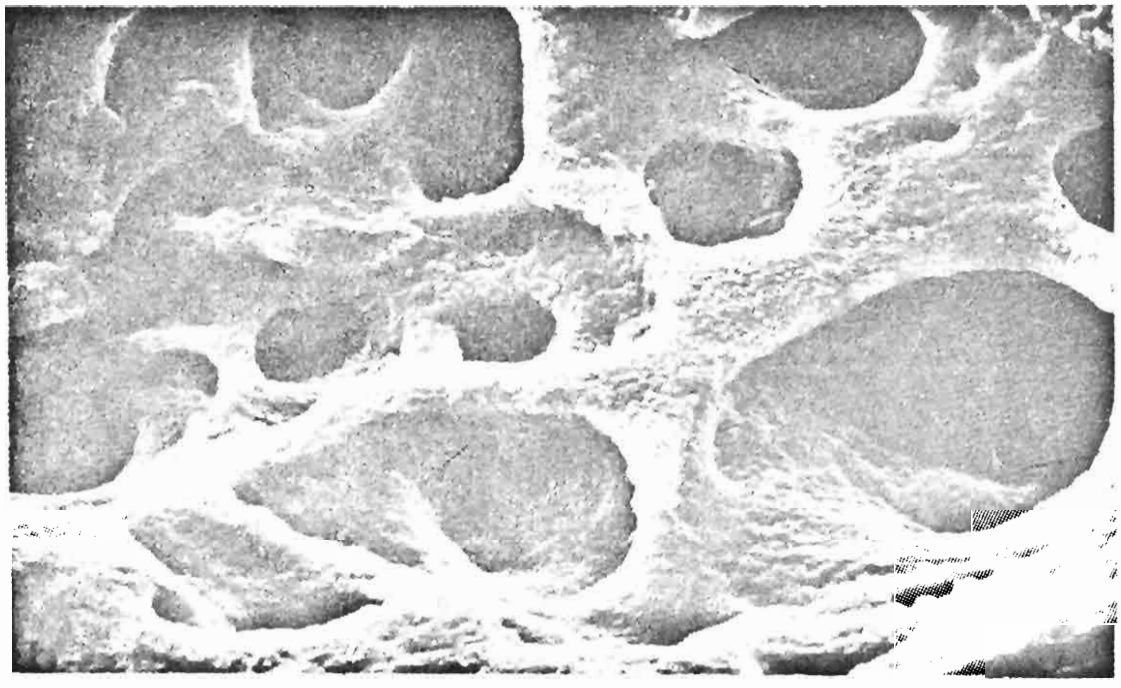
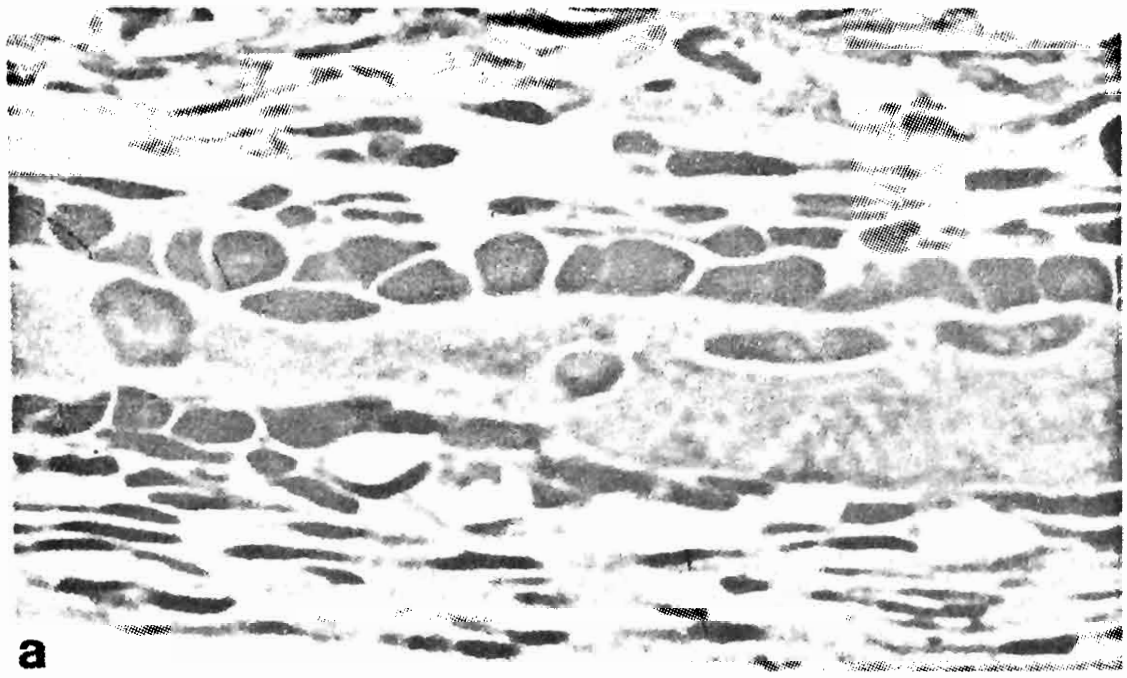


Fig. 7. Sequence of osteoblast isolation. H&E. X 900. a) Intact parietal bone. Note the well developed osteoblasts on the dorsal surface and the flattened cells on the ventral side. b) Parietal bone, periosteum removed (undecalcified). Osteoblasts are left on the dorsal surface; few cells remain ventrally. c) Parietal bone after incubation in collagenase (undecalcified). Of four lacunae present, three contain osteocytes (arrows). Artifact (A).



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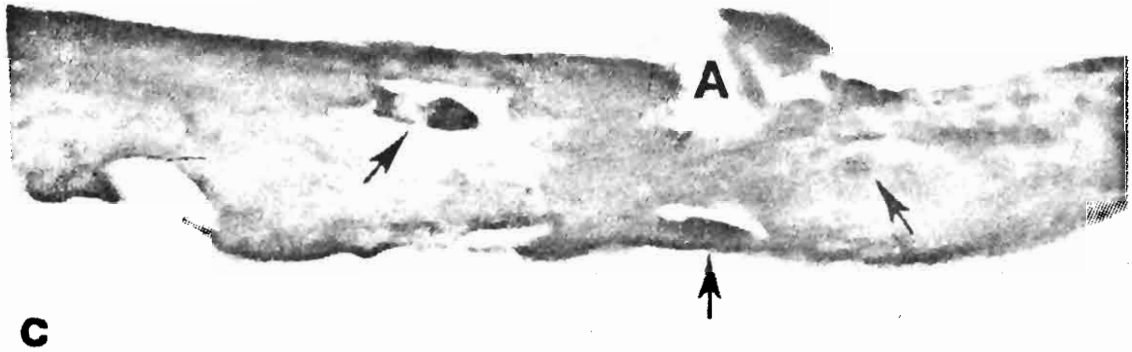

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Fig. 8. Effect of age on cell types found in isolated osteoblast preparations.

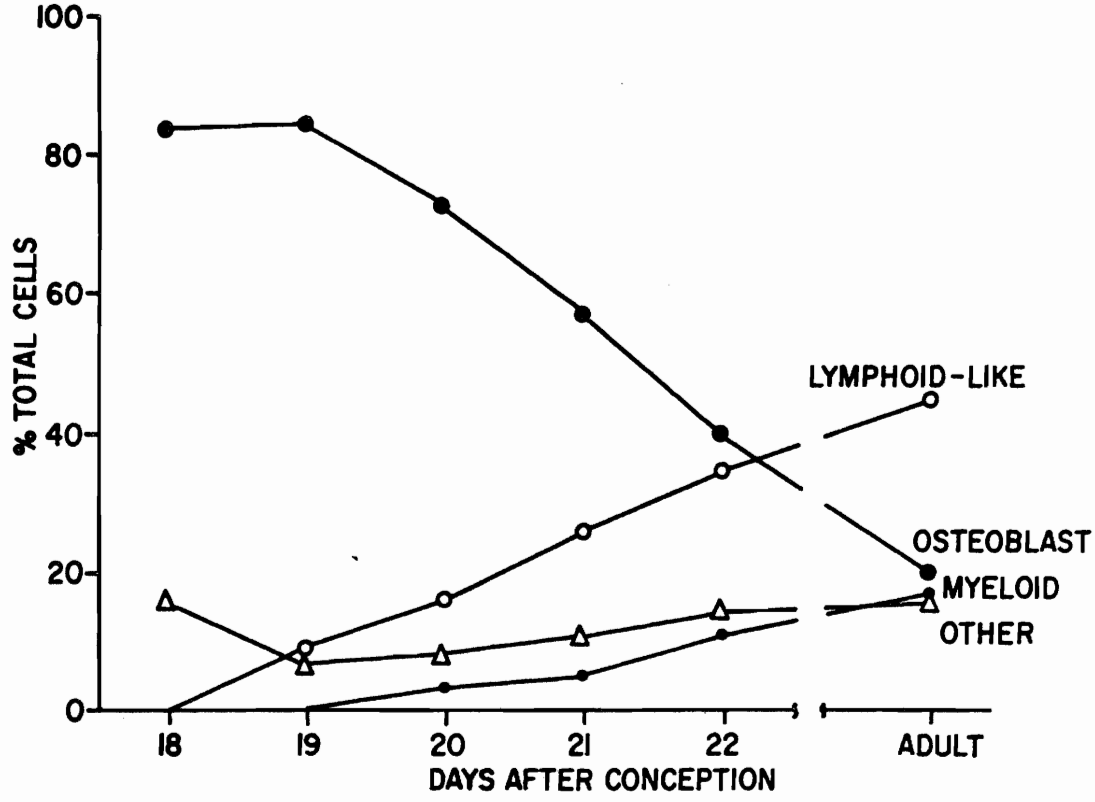
100
80
60
40
20
0
% TOTAL CELLS
LYMPHOID-LIKE
OSTEOBLAST
MYELOID
OTHER
18
19
20
21
22
ADULT
DAYS AFTER CONCEPTION

Fig. 9. Photomicrographic composite of various cells found in isolated cell preparations. Wright's stain. X 2200. a) Osteoblast. b) Active fibroblast. c) Macrophage. d) Lymphoid-like cells (fibrocytes). e) Lymphoid-like cell (small lymphocyte) and erythrocyte. f) Early promyelocyte. g) Metamyelocyte. h) Juvenile neutrophile (stab cell). i) Adult neutrophile. j) Eosinophile. k) Mast cell.

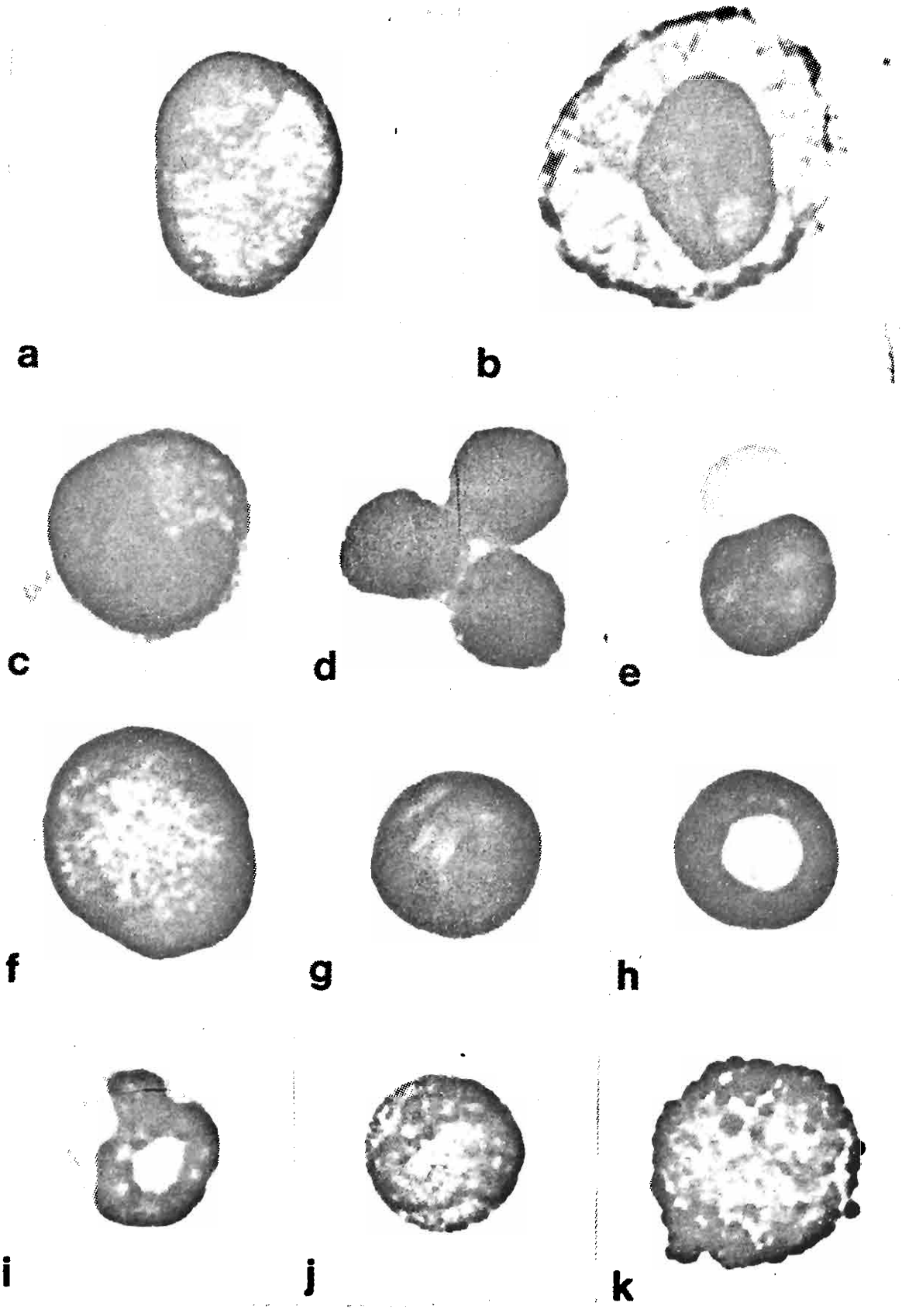
a
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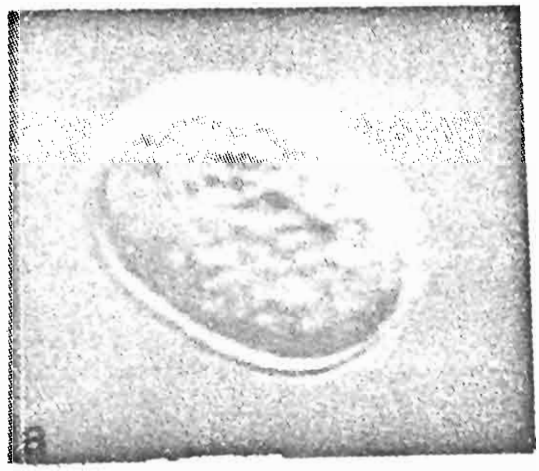
Fig. 10. Nomarski interference contrast. Note the eccentric nuclei and prominent stippling over the golgi regions of osteoblasts. X 2400. a) Osteoblast. b) Lymphoid-like cell. c) Four osteoblasts flattened between coverslips.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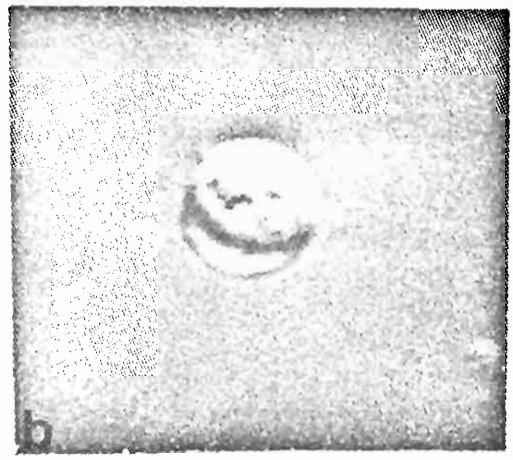
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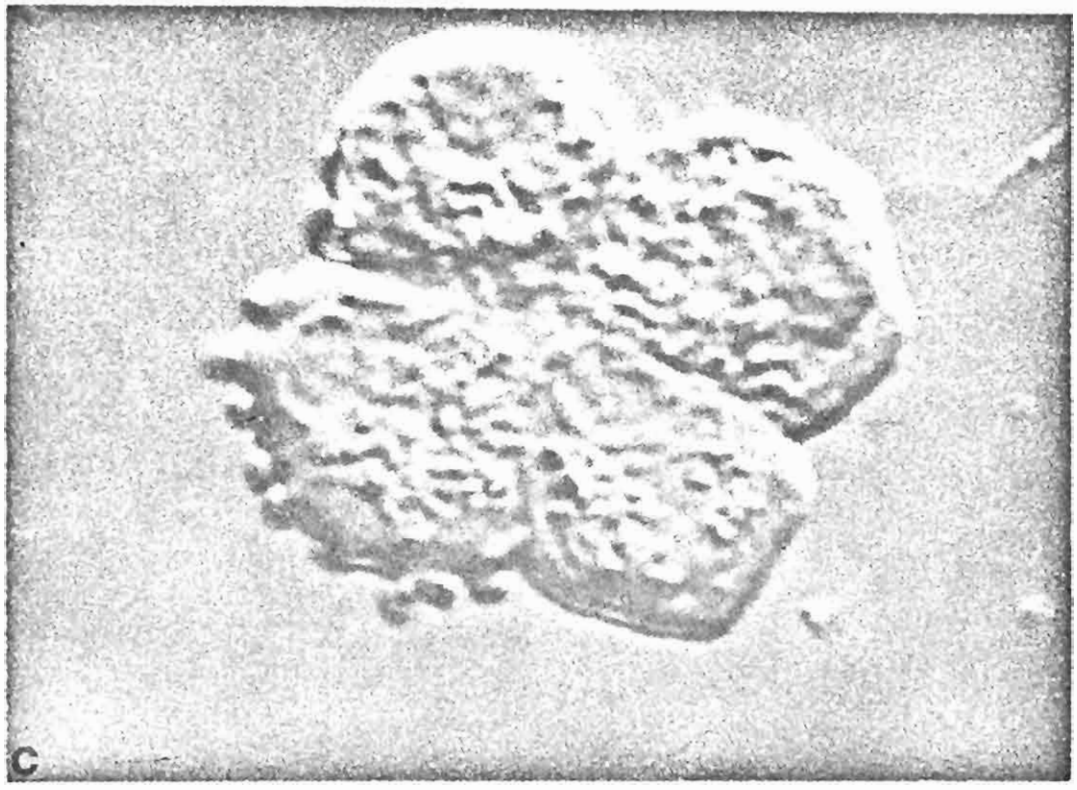
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Table 1. Differential cell counts of selected sites of the rat calvarium

Site	% Osteoblasts	% Osteocytes	% Multinucleated Osteoclasts	% Other Cells	Total Cells Counted
1	0	0	0	100	1221
2	27.7	10.6	0	61.7	698
3	27.4	9.0	0.8	62.8	871
4	25.6	12.3	0.5	61.6	634
5	0	0	0	100	1507
6	20.7	6.6	0	72.7	639
7	18.6	7.6	0	73.8	683
8	19.6	5.4	0	75.0	612
9	0	0	0	100	1287
10	9.2	2.7	0	88.1	1536

Table 2. Cell percentages of the major structures of the rat calvarium

Area	% Total Surface Area	% Total Osteoblasts*	% Total Osteocytes*	% Total Cells
Frontal bone - central area	30.5	48.6 (9.1)	44.5 (3.0)	33.2
Frontal bone - periph. area	8.9	10.7 (2.0)	12.5 (0.8)	7.4
Parietal bone - central area	35.0	29.4 (5.5)	33.6 (2.3)	29.8
Parietal Bone - periph. area	13.5	11.3 (2.1)	9.4 (0.6)	10.6
Sutures	12.1	0	0	19.0
Total calvarium	100.0	100.0 (18.7)	100.0 (6.7)	100.0

* Expressed as a percentage of the total cells in that classification. (Values in parentheses are calculated as a percentage of the total population of cells.)

Table 3. Influence of dissection method on bone cell isolation

Dissection procedure	Total yield (cells/calvarium)	% osteoblasts
Calvarium untouched	2.7×10^6	16
Periosteum removed,* sutures intact	0.9×10^6	25
Present method**	0.04×10^6	85

* Mixed bone cells

** Osteoblast preparation

PART II

INFLUENCE OF CELL ISOLATION METHODOLOGY ON OSTEOBLASTIC CALCIUM DETERMINATIONS

ABSTRACT

Measurements of the calcium content in osteoblasts isolated from 19-day fetal rat calvaria were found to be adversely affected by 3 major factors, including: 1) the presence of calcium-containing particles in the crude collagenase preparation utilized for cell isolation; 2) a calcium-accumulating effect of prolonged collagenase exposure on cell isolates; and 3) the existence of pronounced osseous fragment contamination. These detrimental influences were eliminated respectively by: 1) filtration of collagenase solutions prior to bone incubation; 2) reduction of the collagenase exposure time; and 3) utilization of a mild acid incubation to solubilize osseous fragment mineral. These procedures, in concert, reduced the apparent osteoblastic calcium concentration from approximately 160 to 10 mM. The latter value should be considered only a maximal limit at this time since it is not yet clear if mild acid exposure removes all osseous calcium deposits.

INTRODUCTION

Several models proposed recently to account for short-term, skeletal-dependent calcium homeostasis have included transport through osteoblasts as the obligatory pathway for the removal of calcium from bone (8, 13). Evidence for this was derived in part from morphological examinations which indicated that osteoblasts contained significant quantities of calcium in particulate form (5, 6) and that bound calcium in these cells was sensitive to parathyroid hormone and to calcitonin (7). Biochemical investigations with mixed bone cells have also provided support for the osteoblastic transfer of calcium (2, 3, 8, 9). The use of undefined populations of cells and the possibility of mineralized fragment contamination in these studies, however, make conclusions regarding osteoblastic calcium tentative. The present investigation was an attempt to assess, quantitatively, calcium metabolism in a relatively pure preparation of osteoblasts. During the course of this work, problems were encountered with bone fragments and with the crude collagenase relied upon for enzymatic isolation. These difficulties and some preliminary results concerning osteoblastic calcium are discussed.

MATERIALS AND METHODS

Except as noted, incubations in this study were performed in Earle's balanced salt solution (Grand Island Biological Co.) modified by a reduction of the calcium concentration to 1.25 mM and the addition of 200 mg/ml bovine serum albumin (Fraction V; Miles Laboratories, Inc.). Crude collagenase (lot CLSII 45A154; Worthington Biochemical Corp.) was used for all bone cell isolations.

Preparative methods

Osteoblasts and mixed bone cells were isolated from the calvaria of 19-day fetal Sprague-Dawley rats according to the method of Yagiela and Woodbury (submitted for publication). This isolation procedure, referred to as method 1, was subsequently altered several times during the course of investigation to remove various sources of calcium contamination. Method 2 included the removal of particulate calcium in collagenase solutions by filtration through 0.4μ pore polycarbonate membranes (Nuclepore Corp.) and by a reduction in the incubation volume from 6 to 3 ml. Method 3 constituted a modification in the isolation scheme to limit collagenase exposure time. Bones were incubated in (filtered) collagenase for 30 min (instead of 2 hr), after which the medium and any released cells were discarded

or, on occasion, saved for study. The bones were rinsed in fresh Earle's solution (collagenase free) and incubated for 90 min at 37°C and 100 oscillations/min to remove remaining cells by shaking. Finally, method 4 consisted of suspending cells isolated by method 3 in acidified, ice-cold Earle's medium (pH 6.0) for 10 min to demineralize contaminating bone fragments.

Bone marrow cells were harvested from mother rats. Tibial diaphyses were excised and cleaned of all adhering tissue. Marrow cores were forced out by exerting pressure at the distal ends with a syringe containing Earle's solution. The cells were mechanically separated by repeated aspirations into a Pasteur pipet and were filtered through nylon bolting cloth (35μ mesh NITEX; Tobler, Ernst and Traber, Inc.) to remove undissociated tissue. After cell yield was assessed with a modified Levy chamber, the suspension volumes were adjusted to contain approximately 5×10^6 cells/ml. About 1/2 of all recovered cells were erythrocytes. Osteoblasts were never observed in marrow isolates.

Cellular integrity was determined by nigrosin exclusion (4) and by phase microscopic observation. Approximately 90% of the osteoblasts were intact, irrespective of the particular isolation method employed. Mixed bone and bone marrow cell viabilities were consistently over 95%.

Osseous fragments employed in this work were prepared from

frontal and parietal bones whose cells were previously removed by method 3. The bones were minced in Earle's solution and ground with a mortar and pestle. Only fragments which passed through a 35μ NITEX filter were studied.

Cell volume assessments

Diameters of 100 osteoblasts in suspension were measured with phase contrast microscopy (14.0 $\pm$ 0.1μ; mean $\pm$ S.E.). Individual cell volumes were calculated and averaged on the assumption that each osteoblast was spherical in shape. The resultant value of 1.41 μl/10^6 cells was employed in all osteoblastic calcium concentration determinations.

For comparison, osteoblasts ($3x10^5$ cells) suspended in 20 μl Earle's medium with ^{14}C-antipyrine (1μ Ci/ml) and ^{3}H-inulin (5 μCi/ml) were drawn into a 25μl cylindrical micropipet (Corning Glass Works) and centrifuged at 2200g for 15 min. A packed cell volume of 1.5μl/10^6 cells was arrived at by comparing the length of the osteoblastic pellet with the distance from the micropipet tip to the 25μl graduation. The pipet segment with the sedimented osteoblasts was then broken off and crushed in a scintillation vial containing 10 ml Handifluor (Mallinckrodt Chemical Works). Dual-label counting was performed with a Packard Tri-Carb model 3385 liquid scintillation counter. Quenching was corrected for by the channels-ratio method. Intracellular water, calculated from the difference between the anti-

pyrine and inulin spaces, was 83% of the total cell volume ascertained by cell diameter measurements.

Mixed bone and marrow cell volumes were determined by measuring directly the packed cell masses of $2x10^6$ and $5\text{-}10x10^6$ cells, respectively, after centrifugation in micropipets. For mixed bone cells, a value of 1.70 ± 0.16 $\mu l/10^6$ cells (mean $\pm$ S. E.) was obtained. Marrow preparations were not expressed on a cellular basis because of variations in the fraction of erythrocytes isolated. Instead, a packed cell assessment was made with each experiment.

Calcium determinations

Osteoblast (10^5 cells/tube), mixed bone cell (10^6 cells/tube) and marrow ($5x10^6$ cells/tube) preparations were aliquoted into 3 ml siliconized glass tubes and centrifuged at 400g for 3 min. Supernatants were decanted and the tubes inverted on absorbant paper. After 3-4 min, cell pellets were resuspended in ice-cold, calcium-free Earle's solution and centrifuged for 1 min at 700g. The tubes were again inverted and allowed to drain. A 1 ml volume of stock diluent (0.25 N HNO_3 with 5.9 g/l La_2O_3) was dispensed into each tube. The contents were fully mixed and incubated for 48 hr at 37°C. Just before measurement, the tubes were centrifuged at 400g for 3 min to sediment cellular debris. Calcium concentrations were determined with an Instrumentation Lab model 153 or with a Perkin-Elmer model 303 atomic absorption spectrophotometer. Solutions (without bones)

taken through the entire cell isolation scheme served as blanks. Initial experiments with ^{14}C-inulin as an extracellular marker demonstrated the reliability of this procedure for correcting against soluble calcium contamination.

Radiocalcium uptake

Calcium exchange was monitored by measuring the uptake of ^{45}Ca into osteoblasts captured by passing cellular suspensions through 0.4 μ nuclepore membranes. Histological sections of representative membranes revealed that herniation of cellular material did not occur during filtration. Cell-coated membranes ($3x10^4$ osteoblasts/filter) were incubated in Earle's solution with ^{45}Ca (5μ Ci/ml) for 30 min. Filters were then blotted on the non-cell side, dipped sequentially for 5 sec in each of 3 radiocalcium-free baths and transferred to scintillation vials for counting. Examination of incubation and wash media indicated that cellular adhesion was not adversely affected by these procedures.

Characteristic x-ray analysis

Osteoblasts and marrow cells were collected for scanning electron microscopy by entrapment on nuclepore membranes or by attachment onto collagen-coated plastic petri plates (Falcon products; Becton, Dickinson and Co.). Some cell samples were fixed in 1% osmic acid buffered with Na cacodylate (pH 7.4). Dehydration in all cases was accomplished by quickly passing the preparations over a

Bunsen burner. Specimens were coated with evaporated films of carbon and gold, and cells were viewed with a Cambridge Stereoscan Mark 2 operated at an accelerating volatage of 20 kV. Relative calcium concentrations were assessed by the quantitation of emitted characteristic x-rays with an x-ray energy dispersive analyser (EDAX: Nuclear Diodes, Inc.).

RESULTS

Initial measurements of the calcium content in osteoblasts were questioned when bone marrow cells, exposed to identical isolation regimens, were found to have calcium concentrations several times that predicted from control determinations. Of the various possibilities considered, 3 factors were identified which adversely influenced calcium measurements. These included: 1) the presence of particulate contamination in crude collagenase; 2) a deleterious effect of prolonged collagenase exposure; and 3) the existence of osseous fragments in bone cell preparations.

Collagenase contamination

Several different collagenase samples were obtained and examined for their calcium content, both total and particulate (Table 1). All of the batches tested had considerable quantities of calcium, some of which was insoluble and could therefore interfere with cellular calcium determinations. Marrow cells suspended in Earle's solution with crude collagenase for as little as 5 min were invariably higher in calcium than cells incubated in collagenase-free media. Although the reduction of incubation volumes limited this effect, the problem was solved completely only after collagenase solutions were filtered

through 0.4 μ nuclepore membranes before use. The particulate nature of this contamination and the effectiveness of filtration were observed with phase contrast microscopy (Fig. 1). Insoluble inclusions were of 2 forms - phase-bright spherules and phase-dark, often comma-shaped rods, characteristically thickened at one end. There was a direct correlation between the number of particles and the amount of insoluble calcium in the preparations.

Collagenase exposure time

Although prefiltration of collagenase solutions eliminated problems associated with brief contact, marrow cells incubated for several hours were found to contain excessive amounts of calcium (Table 2). To determine the rate at which calcium was accumulated during exposure, aliquots of marrow cells were measured after various periods of incubation. Cellular calcium values were unchanged at 15 and 30 min, began to rise by 60 min and were markedly increased after 120 min. This effect required the continuous presence of collagenase. Cells exposed to enzymic media for 30 min and then incubated for 90 min in collagenase-free Earle's solution were not different significantly from initial or 30 min controls. To ascertain if these observations were pertinent to bone cells, similar experiments were carried out with mixed bone cells and with osteoblasts harvested after a 30 min incubation of calvaria in collagenase. Results were essentially the same as described for marrow cells

(Table 2).

Since cells exposed to collagenase for brief periods of time were unaffected during subsequent suspensions in enzyme-free solutions, the isolation of osteoblasts by an abbreviated collagenase digestion was attempted. The release rate of osteoblasts from bone was determined by counting cells in samples of media collected over a 4 hr collagenase incubation period (Fig. 2). More than 1/2 of the total cells isolated were freed by 30 min; however, these cells were unsuitable for routine study as considerable amounts of cellular debris were also evident microscopically. The problem was eventually solved by discarding this 30 min suspension and re-incubating the bones in fresh, collagenase-free media for 90 min more (method 3). The additional shaking dislodged about 16,000 cells/calvarium, a figure similar to that obtained with collagenase digestion during the same period (30-120 min, Fig. 2). Furthermore, debris was minimized by this procedure, and osteoblast purity was improved to 90%.

Bone fragments

An independent assessment of osteoblastic calcium was deemed necessary after the difficulties with enzymatic incubation were encountered. From osteoblasts and marrow cells prepared for scanning electron microscopy (Fig. 3), relative calcium concentrations were obtained by quantifying the number of characteristic x-rays emitted from the cells during exposure to the scanning electron beam

(Fig. 4a). X-ray spectra indicated that osteoblasts contained significantly more calcium than did erythrocytes and marrow cells. Isolated bone fragments, identified by their appearance (Fig. 3c, d) and high mineral content (Fig. 4b) were observed, however, in bone cell preparations. It was apparent that this was a potential source for calcium contamination.

Recently, Dziak and Brand reported that mild acid exposure decalcified osseous fragments in mixed bone cell preparations without affecting cellular function (2). Table 3 illustrates our preliminary experience with this method. The calcium concentration of marrow cells was unchanged by suspension in ice-cold Earle's solution at a pH of 6.0. Calcium in fragments prepared from bones incubated according to method 3 was reduced by 93% after a 10 min exposure. Finally, it was obvious from the precipitous drop in mixed bone cell calcium that most of the mineral content in that preparation was extracellular.

Summary of findings

Various modifications of the original isolation scheme were successful in reducing the calcium contamination of osteoblast preparations as summarized in Table 4. Alterations in total as well as exchangeable calcium parameters were noted, with ^{45}Ca-uptake determinations apparently more affected than calcium concentration measurements.

DISCUSSION

Enzymatic digestion of fetal bone, in contrast with other tissues, is a slow and arduous process. Incubations from 90-120 min are generally required, and collagenase preparations containing significant proportions of other proteolytic activities are often chosen to obtain maximal cell release (2, 10, 12). One could reasonably assume, therefore, that the accumulation of calcium observed here during prolonged enzymatic contact might be related to cellular injury. Several findings dispute this, however. Nigrosin uptake into marrow cells was not increased over initial control values by a 2 hr incubation. The cellular integrity of osteoblasts isolated by method 2 was indistinguishable from that calculated for cells harvested by method 3. Finally, previous work with mixed bone cells indicated that plasma membranes remain functionally intact after prolonged collagenase esposure (2, 3, 10, 12). Even if cells were damaged by incubation, it would be erroneous to conclude that the calcium accretions were causally related. Increases similar to those observed in marrow preparations were also obtained with mixed bone cell isolates, where 87% of the initial total calcium was located extracellularly.

It is possible, inasmuch as only a single collagenase preparation

was used for incubation, that the influence on cellular calcium was limited to the particular batch employed. As new lots of the Worthington blend especially suited for bone (type II) become available, they will have to be studied. Two lines of support, however, suggest that the effect may not be unique. Firstly, we found relatively large amounts of calcium associated with each of the various collagenase samples examined, even after removal of particulate inclusions. A very small percentage of the calcium-containing substance in any of the preparations would be sufficient, when precipitated with cells during centrifugation, to seriously impair calcium determinations. Secondly, Bray and Clark reported a confirming observation (1). In attempts to remove organic debris interfering with measurements of calcium influx in isolated intestinal cells, those investigators tested a wide variety of enzymes in hopes of finding one that would selectively eliminate unwanted ^{45}Ca accumulation. Of 13 preparations studied, several proteolytic enzymes were successful in reducing the binding of ^{45}Ca to damaged cells. Collagenase, surprisingly, had the opposite effect, and calcium uptake was increased markedly.

The mechanism by which collagenase affects the calcium content of cell isolates is not yet understood. Since cell-free collagenase solutions remain unaltered during prolonged incubation and cells incubated in the absence of collagenase do likewise, it appears that

some interaction between the cell preparation and one or more constituents in crude collagenase is essential. Perhaps a proteolytic enzyme affects some calcium-binding moiety such that it adheres to and sediments with cells and other precipitable material. A similar adhesion may explain why only portions of contaminating inclusions in unfiltered collagenase preparations can be removed by low-speed centrifugation. The rest probably settle only in association with cellular elements.

Before problems with crude collagenase and bone fragments were encountered, the objective of this work was to describe, quantitatively, the mineral metabolism of the osteoblast - a cell reportedly unique in its sequestration of enormous amounts of calcium (8). During attempts to remove all sources of contamination, the calcium concentration in osteoblast isolates fell by almost 95%, and a similar effect was observed with mixed bone cell preparations. Although it is premature to question whether osteoblasts, in fact, contain exceptional quantities of calcium, it is reasonable to reexamine some ot the reported evidence for this.

Much of the biochemical support for a high calcium content in bone cells was obtained by Nichols and his associates (8, 9). Calcium values between 100 and 200 mM were calculated for cells harvested mechanically from trabecular as well as cortical bone. Although these workers have performed a number of experiments in attempts

to prove otherwise, it appears, as recently contended (2), that their results reflect, primarily, mineral associated with bone fragment inclusions. Actually, some of Nichols' data can be interpreted as demonstrating osseous contamination. For example, marrow cells obtained from washings of minced trabecular bone were reported to contain 15 times as much calcium as was associated with our marrow preparations (9). Nichols and Rogers attributed excessive calcium in marrow isolates to the presence of osteoblasts. Histological examinations were not performed, however, and most of the extraneous calcium likely came from bone chips formed during mincing. In other work, Nichols et al. "confirmed" their bone cell calcium results by showing that trabecular bone cells, harvested from a suckling pig 15 min after a tetracycline injection, contained 10 times more drug than did marrow cells (8). Assuming that tetracycline binding provided an accurate measure of cellular calcium in this experiment and that the bone cells were, in fact, mostly osteoblasts (or osteocytes), these data would just as easily confirm our findings.

Perhaps more germane to the present study are the investigations by Dziak and Brand (2,3). Using procedures specifically designed to eliminate bone fragment contamination, these workers found that mixed bone cells isolated from fetal rat calvaria by a 2 hr incubation in crude collagenase contained 16.8 mmoles of calcium per kg wet weight. Although this estimate of bone cellular calcium was lower

than any previously determined, it is almost 10 fold higher than that reported here with an analagous mixed cell preparation. We believe the effect of prolonged collagenase exposure on cellular calcium measurements may account for the difference in results.

Conclusions at this point regarding the calcium content of osteoblasts should be guarded. Although the analysis of calcium-specific x-rays indicates that osteoblasts contain more calcium than do marrow cells, quantitative consideration of these data is hazardous because of the limited sampling volume of the exciting scanning electron beam. The atomic absorption determination of 10.2 mM, at present, provides essentially an upper limit for the true calcium concentration. This value was calculated on the assumption that acid exposure completely demineralized all contaminating osseous fragments. If, for example, 7% of the bone fragment calcium actually remained unsolubilized, the osteoblast concentration would be 5.6 mM. Experiments are currently underway to provide an accurate assessment of mineral metabolism in these cells. From the results reported herein, however, it may be concluded that osteoblasts, at least those present in fetal rat calvaria, do not contain the enormous concentrations (i. e. 150 mM) of calcium sometimes ascribed to them and that model systems for the cellular transport of mineral in bone must accomodate a working cell of much more moderate calcium content.

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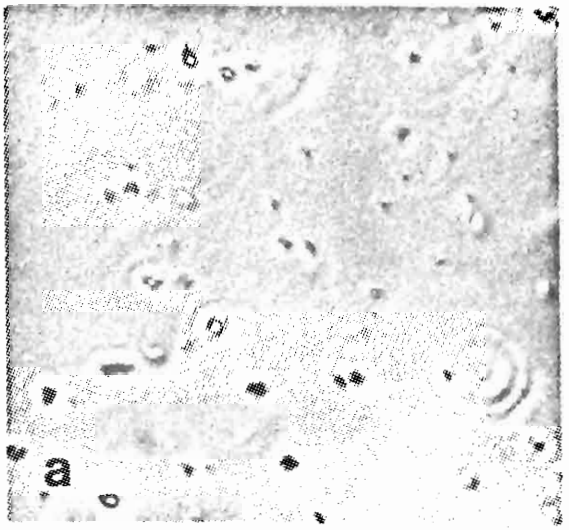
Fig. 1. Removal of particulate collagenase contaminants. Phase contrast. X 725. a) Collagenase (batch CLS 45B194) dissolved in Earle's solution, 2.5 mg/ml. b) Collagenase solution after nucleopore filtration.


a

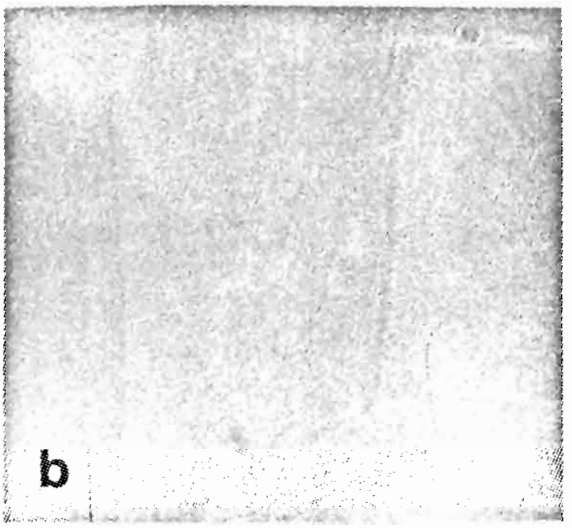

b

Fig. 2. Rate of osteoblastic release from bone during collagenase incubation.

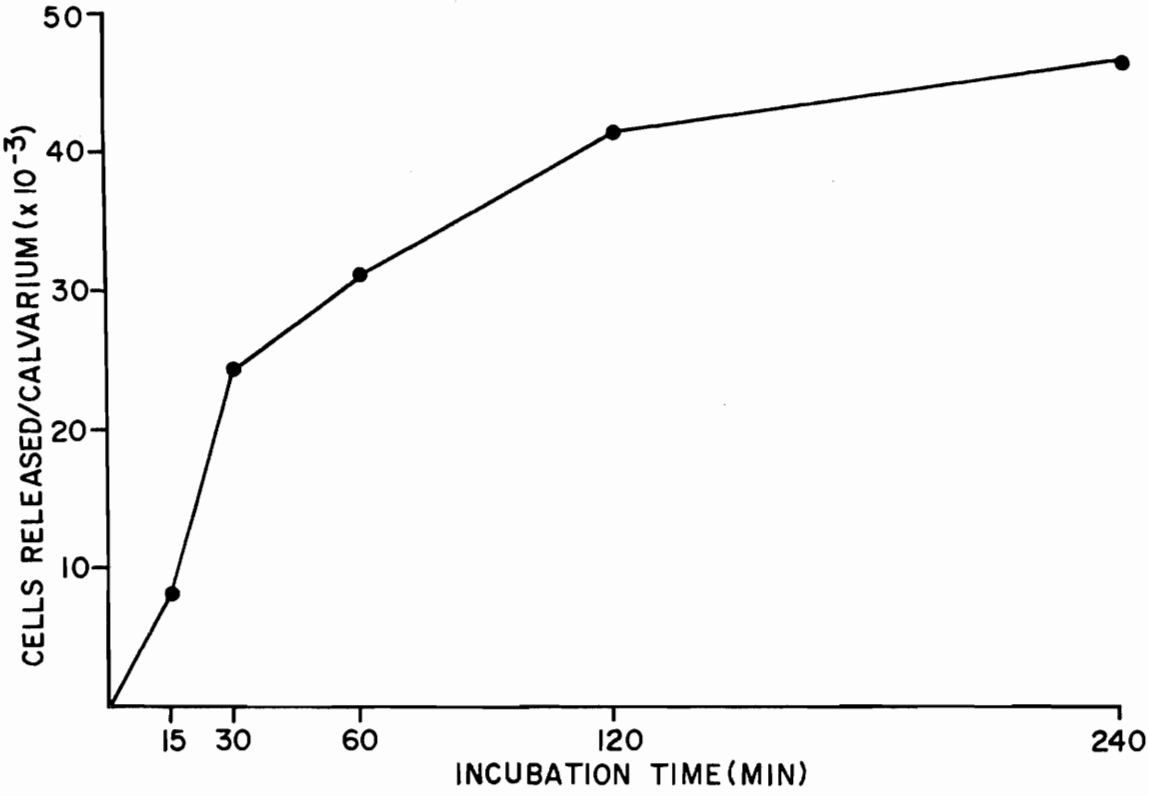
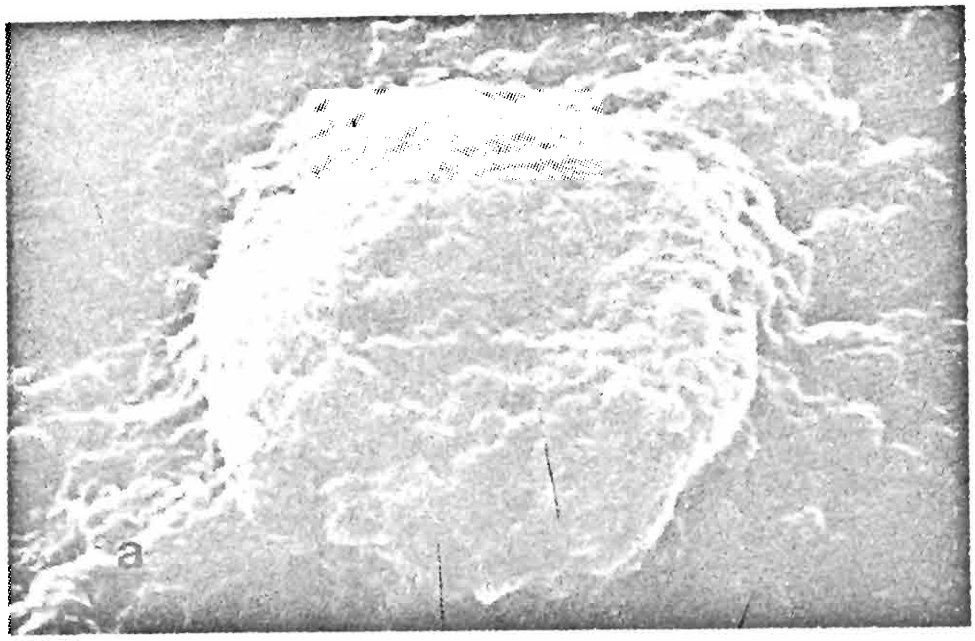

50
40
30
20
10
CELLS RELEASED/CALVARIUM (x 10⁻³)
15
30
60
120
240
INCUBATION TIME (MIN)

Fig. 3. Scanning electron micrographs of cell preparations. X 5500. a) Osteoblast. b) Marrow cells. c)Bone fragment trapped on nuclepore filter. d) Osteoblast with adherent bone chip (arrow).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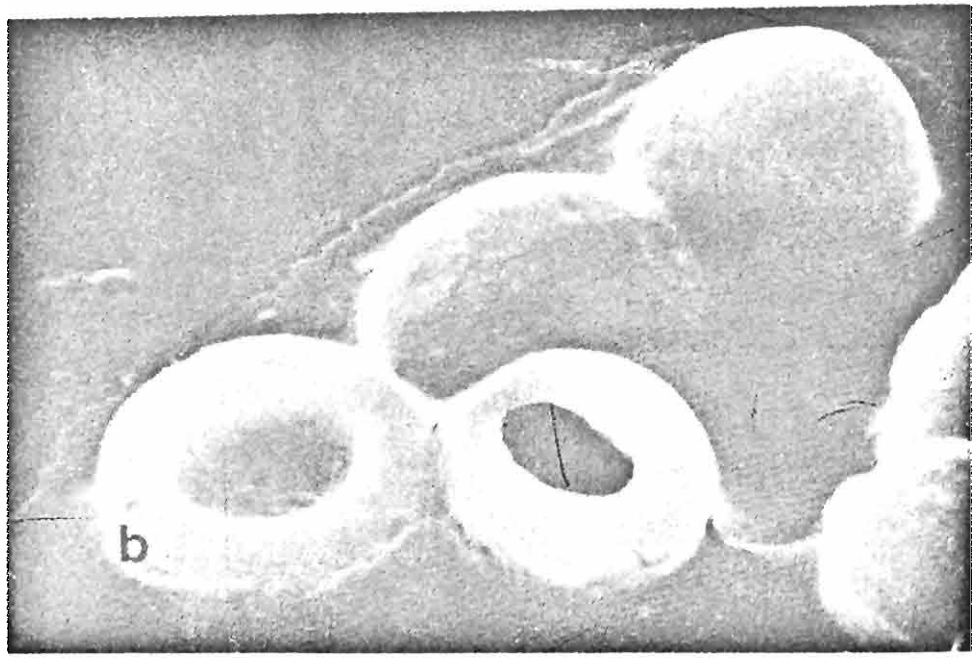
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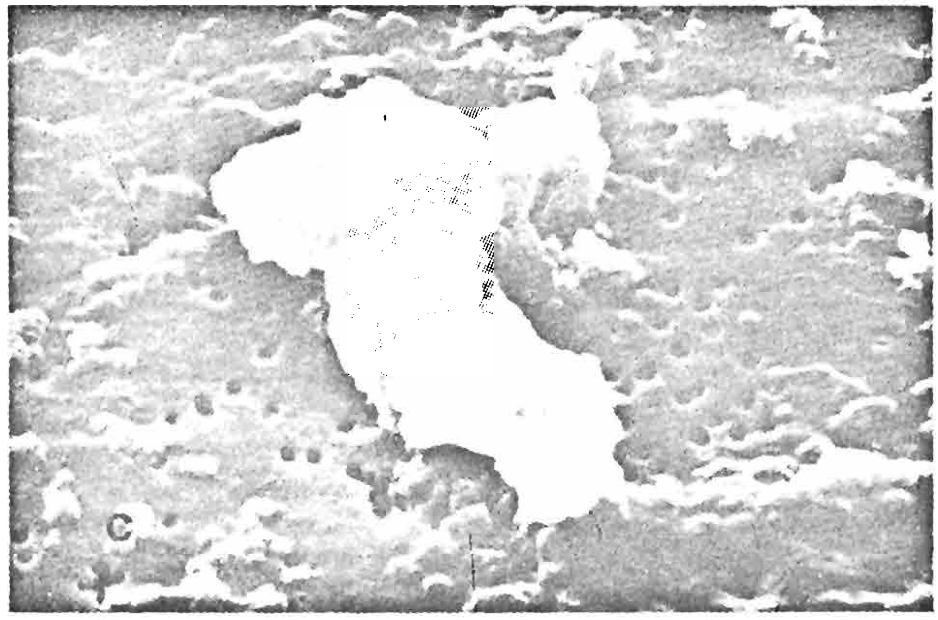
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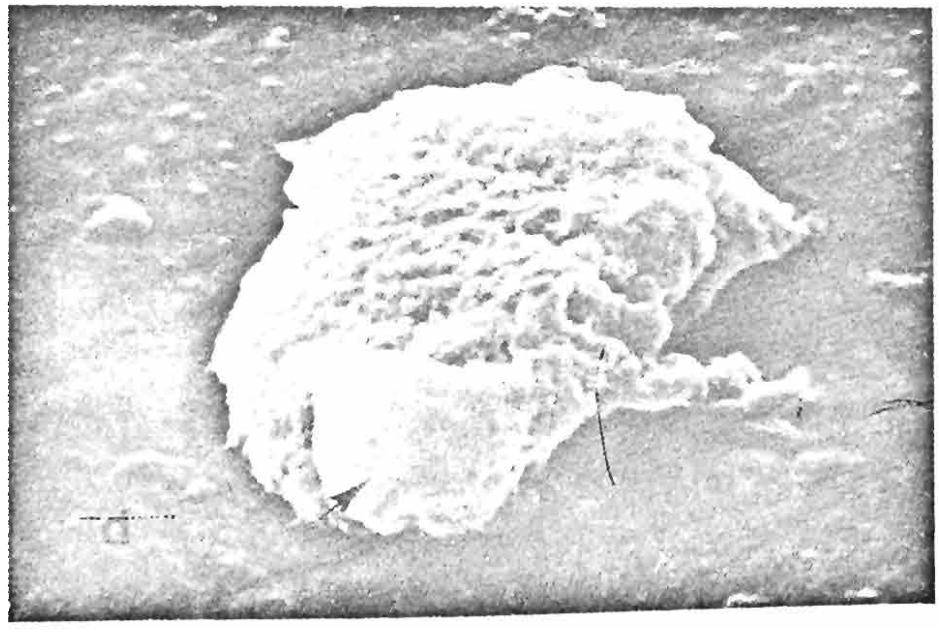
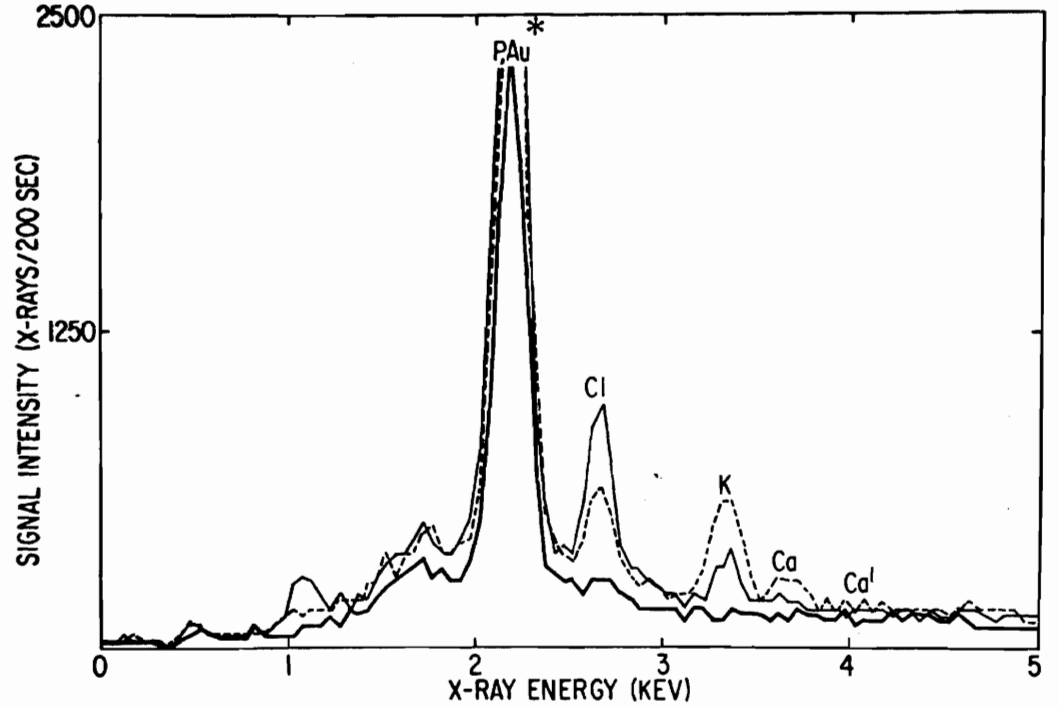

D

Fig. 4. Characteristic x-ray spectra. The background spectrum is depicted by the thick continuous line. Ca' marks the secondary calcium emission peak. * Gold and phosphorus peaks were not quantified because of the considerable overlap. a) Dashed line - osteoblast; thin, solid line - erythrocyte. b) Dashed line - bone fragment; thin, solid line - scraped fetal bone. ** Calcium peak of fetal bone was 3450 x-rays/200 sec.


2500
1250
SIGNAL INTENSITY (X-RAYS/200 SEC)
*
P,Au
Cl
K
Ca
Ca'
0
1
2
3
4
5
X-RAY ENERGY (KEV)

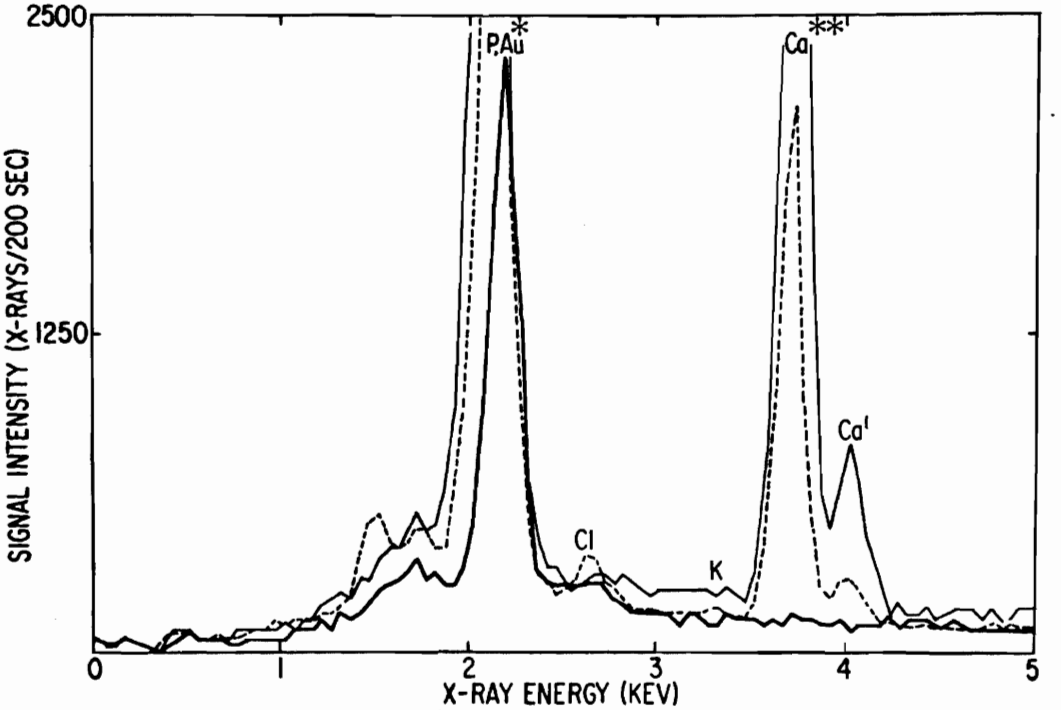

2500
1250
SIGNAL INTENSITY (X-RAYS/200 SEC)
*
P,Au
**'
Ca
Ca'
Cl
K
0
1
2
3
4
5
X-RAY ENERGY (KEV)

Table 1. Calcium content of various collagenase preparations

Batch I.D. (Worthington)	Collagenase Activity (units/mg prep.)	Other Enzymes Present	Ca Content (nm/mg prep.)	Ca/Collag. (nm/u act.)	Particulate Ca (nm/mg prep.)
CLSP4 54K405	459	-	81.8	0.18	None
CLS 45B194	163.5	++	29.7	0.18	1.44
CLSII 44M196	139	+++	21.1	0.15	**
CLSII 45A154	195	+++	28.5	0.15	0.76
CLSIII 44K185	125	+	49.5	0.40	0.68
CLSIV 44N288	200	+++,+	13.0	0.07	0.14

- essentially no other enzymes present

++ standard commercial mixture of proteolytic enzymes (including caesinase, clostriopeptidase and tryptic activities

+++ high in proteolytic enzymes

+ low in proteolytic enzymes

+++,+ high in caesinase, low in tryptic activities

Table 2. Effect of collagenase incubation on cell calcium

Cell Type	0 min (mM ± S.E.)	30 min (mM ± S.E.)	120 min (mM ± S.E.)	% Increase (30 - 120 min)	30 min + 90 min (mM ± S.E.)
Marrow Cells	0.88 ± 0.05 (9)	0.92 ± 0.21 (3)	1.62 ± 0.26 (5)	98 ($p<.05$)	
Marrow Cells	0.85 ± 0.08 (4)				0.80 ± 0.06 (4)
Mixed Bone Cells		18.3 ± 5.3 (4)	37.8 ± 2.9 (4)	107 ($p<.05$)	24.0 ± 5.5 (4)
Osteoblasts		99 ± 25 (6)	147 ± 28 (6)	48 ($p<.05$)	

Number of observations per experiment are shown in parentheses. Significance evaluations were performed with Student's t-test. Blank spaces indicate that values were not determined.

Table 3. Effect of pH 6 incubation on calcium content

Preparation	Control	Treated	% Change
Marrow cells	0.79 ± 0.08 (4)	0.80 ± 0.05 (4)	+ 1
Mixed bone cells	14.4 ± 0.7 (5)	1.8 ± 0.5 (2)	- 87
Bone fragments	3195 ± 8 (6)	234 ± 0 (6)	- 93

Cellular calcium values are expressed in μmoles/ml cell volume ± S.E.; bone fragment calcium is shown in μmoles/g wet weight ± S.E. The number of observations per experiment are indicated in parentheses. Treated groups were suspended in ice-cole Earle's solution, pH 6.0, for 10 min. Controls were incubated at pH 7.4 and 37°C.

Table 4. Reduction of calcium is osteoblast preparations by modifications of the isolation procedure

Isolation Method	Ca Content (mM ± S.E.)	^{45}Ca Uptake (DPM/10^4 cells ± S.E.)	Ratio (DPM x 10^{-6}/mmole Ca)
1	158.6 ± 21.1 (7)	4853 ± 517 (6)	2.17
2	96.4 ± 11.5 (4)	**	**
3	71.6 ± 5.7 (6)	1906 ± 226 (4)	1.89
4	10.2 ± 2.6 (2)	215 ± 20 (4)	1.49

** Not determined

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Professional Organizations

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